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PHYSIOLOGICAL AND ULTRASTRUCTURAL STUDIES ON THE LONGITUDINAL RETRACTOR MUSCLE OF A SEA CUCUMBER *STICHOPUS JAPONICUS*

I. FACTORS INFLUENCING THE MECHANICAL RESPONSE

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SUMMARY

1. The physiological properties of contraction and the ultrastructure of the longitudinal retractor muscle (LRM) of a sea cucumber *Stichopus japonicus* were studied to give information about the sources of activator Ca in echinoderm somatic smooth muscles.

2. The magnitude of ACh- and K-induced contractures was dependent on $[Ca]_o$, and both contractures were eliminated in Ca-free solution, while they were not markedly influenced by Mn ions (10 mM) and low pH (4·0).

3. Procaine (5 mM) decreased ACh-contracture tension with a long lasting after-effect, which was removed by the application of high $[K]_o$.

4. ACh-contractures were markedly potentiated shortly after the termination of mechanical response to the removal of external Na.

5. The LRM could also be made to contract by caffeine (10 mM), the removal of external divalent cations and hypertonic solutions, indicating the presence of intracellularly stored Ca available for the activation of the contractile mechanism.

6. The LRM fibres contain poorly developed intracellular membranous structures. The inner surface of the plasma membrane and small vesicles located underneath the plasma membrane seem to be two of the sources of activator Ca.

INTRODUCTION

Echinoderms are an interesting group of animals, since they seem to have a common evolutionary origin with the chordates while their relation to other invertebrates is obscure.

It has been well established that the contraction–relaxation cycle in vertebrate fast striated muscle is regulated by the release of Ca from, and its uptake by, the sarcoplasmic reticulum (SR) (Ebashi & Endo, 1968). In various kinds of smooth muscle, the sources of Ca activating the contractile mechanism (activator Ca) still remain to be investigated, though some intracellular structures such as the SR and the mitochondria are also known to accumulate Ca (Somlyo & Somlyo, 1976).

Recently, combined physiological, ultrastructural and histochemical studies have been made on some vertebrate and invertebrate smooth muscles (Sugi & Yamaguchi, 1976; Atsumi & Sugi, 1976; Sugi & Daimon, 1977; Sugi & Suzuki, 1978; Suzuki & Sugi, 1978), and have proved very useful in studying intracellular Ca translocation during mechanical activity. The experiments to be described in this and the following paper are concerned with the possible sources of activator Ca in echinoderm somatic muscle, using a sea cucumber *Stichopus japonicus* as material.

MATERIALS AND METHODS

Preparation

Sea cucumbers (*Stichopus japonicus*) with relaxed body length of 20–25 cm were collected at the Misaki Marine Biological Station. The longitudinal retractor muscle (LRM) was carefully isolated from the inner surface of the body wall, and all physiological experiments were performed with a small strip of the LRM (0·5–1 mm in diameter and 0·8–1·2 cm in length). A pair of stainless-steel wire connectors (0·2 mm in diameter and 5 mm in length, Sugi, 1972) were tied to both ends of the preparation with braided silk thread, and the preparation was mounted horizontally at its slack length in an acrylite experimental chamber (5 ml) filled with the experimental solution; one end of the preparation was attached to a lucite hook fixed to the bottom of the chamber, while the other end was connected to a strain gauge (Shinko; compliance, 1 μm/g) to record isometric mechanical responses on an ink writing oscillograph.

All experiments were performed at room temperature (20–26 °C).

Solutions

The standard experimental solution (artificial sea water) had the following composition (mM): NaCl, 513; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 50 (pH adjusted to 7·0–7·2 by $NaHCO_3$). High-K solutions were prepared by substituting K ions (20–400 mM) for equal amounts of Na ions. When external Ca and Mg ions were decreased or removed, an osmotically equivalent amount of Na ions was added. Na-free solution was prepared by replacing all Na ions by choline.

Solutions were perfused at a rate of 50–100 ml/min with a water-vacuum suction tube. When the factors influencing the mechanical response to acetylcholine (ACh), high $[K]_o$ or other contracture-inducing agents were examined, the preparation was previously equilibrated for 30 min in modified standard solutions providing the same experimental conditions as those of the contracture-inducing solutions, unless otherwise stated.

Electron microscopy

The LRM preparation at its slack length was fixed with a 2·5% glutaraldehyde solution containing 0·6 M sucrose and 2 mM-$CaCl_2$ (pH 7·2 by 0·1 M cacodylate buffer). The tissue was then cut into small pieces, postfixed in 2% OsO_4 (unbuffered), dehydrated with a graded series of ethanol, and embedded in Epon 812. Ultrathin sections were cut on a Porter–Blum MT-2 ultramicrotome with a diamond knife, double stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12AS electron microscope.

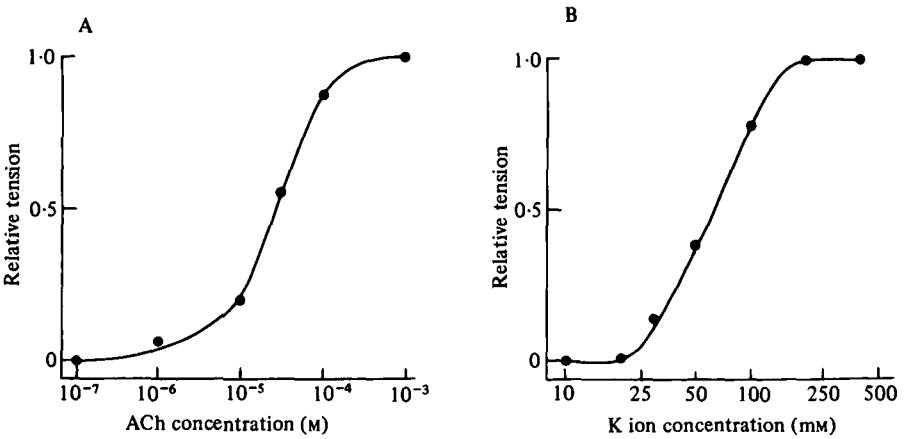

Fig. 1. Relation between the peak height of contracture tension (relative value) and ACh (A) or K ion (B) concentration.

RESULTS

Mechanical responses to ACh and high $[K]_0$

The LRM preparation could be made to contract by ACh (10^{-7} to 10^{-3} M), which is generally believed to be the transmitter in the neuromuscular system of echinoderms (Welsh, 1966; Pentreath & Cobb, 1972; Florey, Cahill & Rathmayer, 1975), or by high $[K]_0$ (20–400 mM). In both cases, the mechanical response was tonic in character; the isometric tension normally showed very gradual relaxation during prolonged application of ACh or high $[K]_0$. The relation between ACh or K ion concentration and the height of resulting contracture tension is shown in Fig. 1. Under the standard $[Ca]_0$ of 10 mM, the maximum ACh-induced contracture tension was obtained with 10^{-4} to 10^{-3} M ACh, while the maximum K-induced contracture tension was attained with 200–400 mM-K. In the same preparation, the maximum tension with ACh was 10–20% smaller than that with high $[K]_0$.

Factors influencing ACh- and K-induced contractures

In order to give information about the degree of contribution of Ca-influx to the activation of the contractile mechanism in the LRM, the effect of some factors on the contracture induced by ACh (10^{-3} M) or high $[K]_0$ (200 mM) was studied.

Change in $[Ca]_0$

A reduction of $[Ca]_0$ from 10 to 1 mM decreased the height of both ACh- and K-contractures by 50–70% (Fig. 2A, B). When Ca ions were removed from the standard solution, the mechanical response to ACh and high $[K]_0$ disappeared completely. In a Ca-free solution containing 5 mM EGTA, the disappearance of mechanical response took place within 3–5 min; on return to the standard solution, the mechanical response to ACh and high $[K]_0$ was restored within 3–5 min. On the

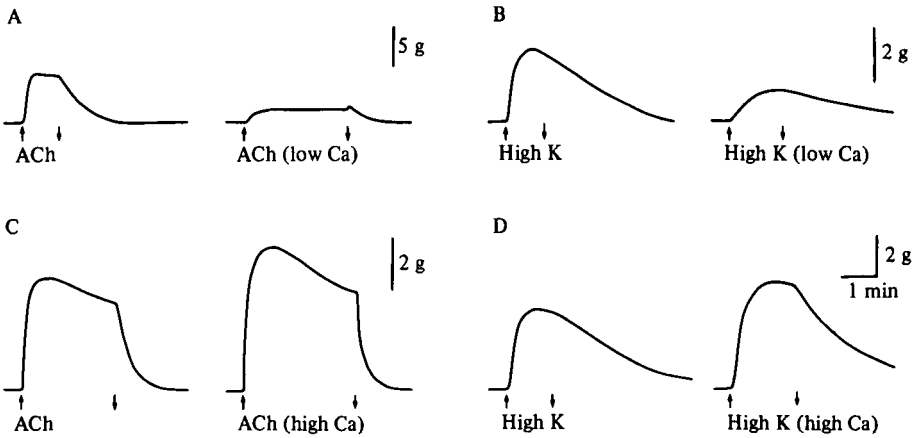

Fig. 2. Dependence of ACh- and K-induced contractures on $[Ca]_o$. (A, B) Decrease of ACh- (A) and K-contracture (B) tension by a reduction of $[Ca]_o$ from 10 to 1 mM. Left traces are control contractures with 10 mM-Ca, and right traces are contractures with 1 mM-Ca. (C, D) Increase of ACh- (C) and K-contracture (D) tension by an increase of $[Ca]_o$ from 10 to 50 mM (substituted for Na). Left traces are control contractures with 10 mM-Ca, and right traces are contractures with 50 mM-Ca.

other hand, an increase in $[Ca]_o$ from 10 to 50 mM (substituted for Na) was found to further increase the contracture tension in response to supramaximal concentration of ACh or K-ions (Fig. 2C, D).

These results indicate that external Ca ions are essential for the activation of the contractile system by ACh or high $[K]_o$, and that the degree of activation is largely dependent on the gradient of Ca ions across the plasma membrane.

Mn ions and pH

In the presence of Mn ions (10 mM, substituted for Na), the height of ACh-contractures was reduced only slightly (by 10–20%), while that of K-contractures was also not so marked (30–40%) (Fig. 3A, B).

Both ACh- and K-contractures were not significantly affected by lowering the pH of the external medium from 7·2 to 4·0 by adding HCl.

Procaine

Procaine (5 mM) reduced the ACh-contracture tension by 50–70%, whereas the reduction of K-contracture tension was less marked (20–30%) (Fig. 3C, D). The inhibitory action of procaine on ACh-contractures was long lasting, a period of 30–60 min being required for complete recovery of ACh-contractures in the standard solution. The recovery process appeared to be markedly accelerated by the application of high $[K]_o$ (Fig. 3E), though this effect was not studied quantitatively.

Other factors producing mechanical response

Experiments were carried out to examine whether the LRM can also be made to contract by some factors which are known to produce contraction in various types of muscle.

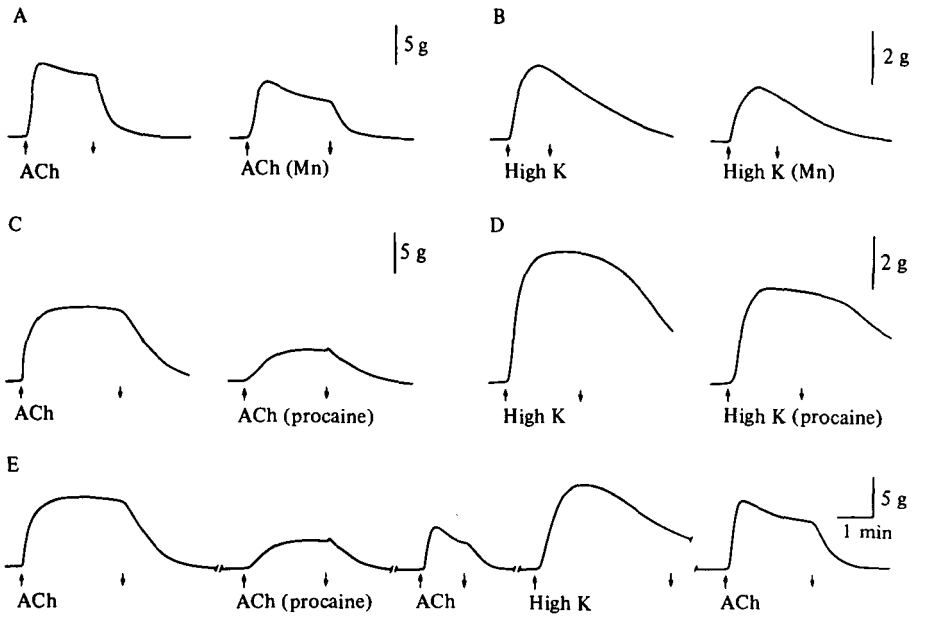

Fig. 3. Effect of Mn ions and procaine on ACh- and K-contractures. (A, B) Decrease of ACh- (A) and K-contracture (B) tension by 10 mM-Mn. Left traces are control contractures, while right traces are contractures in the presence of Mn ions. (C, D) Decrease of ACh- (C) and K-contracture (D) tension by 5 mM procaine. Left traces are control contractures, while right traces are contractures in the presence of procaine. (E) Accelerating effect of high $[K]_o$ (200 mM) on the recovery of ACh-contractures after previous application of procaine (5 mM). Contracture tension in response to ACh (10^{-3}) quickly restores its initial value by an intermittent application of 200 mM-K.

Caffeine

Caffeine (10 mM) produced contracture tension which amounted to 20–30% of the maximum K-induced contracture tension in the presence of 10 mM-Ca (Fig. 4A) or after the removal of $[Ca]_o$. As with molluscan smooth muscle (Twarog & Muneoka, 1972; Sugi & Yamaguchi, 1976), an interval of more than 1 h was required to produce a consistent mechanical response to caffeine in the presence of 10 mM-Ca. When caffeine was applied at shorter intervals, the resulting contracture tension decreased each time of caffeine application. In agreement with Hill (1981), the time required for the complete recovery of the mechanical response to caffeine could be markedly shortened by the application of high $[K]_o$. The mechanical response to caffeine was almost completely inhibited by procaine (5 mM).

Removal of external Ca and Mg ions

When external Ca and Mg ions were removed from the standard solution, the LRM preparation exhibited a slow irregular development of tension which also amounted to 20–30% of the maximum K-contracture tension (Fig. 4B). The rate of tension development was most marked if the Ca-, Mg-free solution contained EGTA (5 mM). The preparation relaxed on return to the standard solution, and

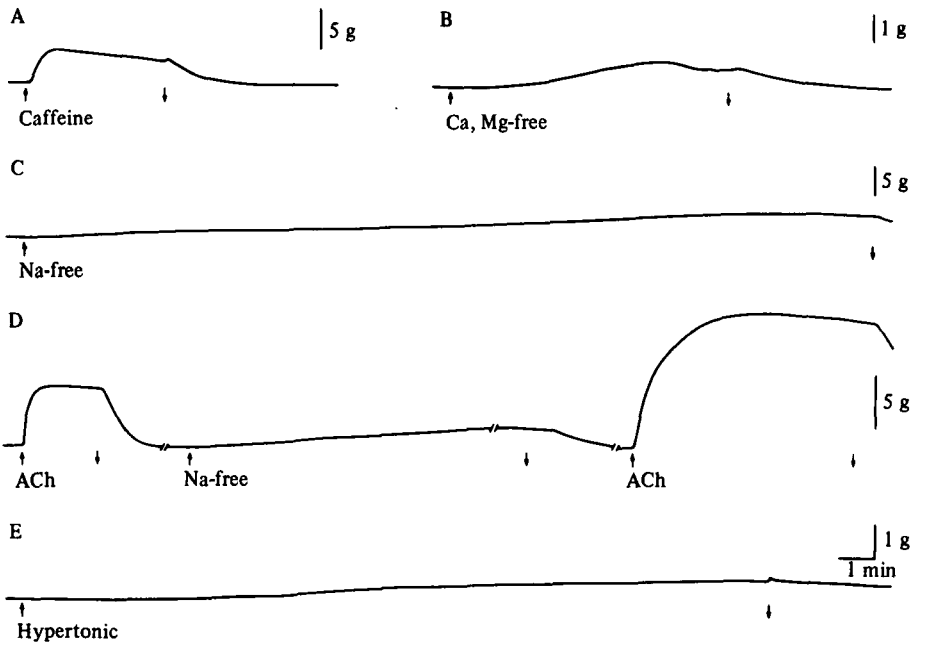

Fig. 4. Mechanical responses of the LRM to various factors other than ACh and high $[K]_o$. (A) Mechanical response to caffeine (10 mM). (B) Mechanical response to the removal of external Ca and Mg ions. (C) Mechanical response to the removal of external Na ions. (D) Increase of ACh-contracture tension shortly after the termination of mechanical response to the Na removal. Control ACh-contracture is also shown. (E) Mechanical response to hypertonic solutions prepared by adding NaCl (613 mM).

similar mechanical responses could be produced many times with repeated application of the Ca-, Mg-free solution at short intervals (3–5 min). Procaine (5 mM) had no marked effect on the mechanical response to the removal of external Ca and Mg ions.

Removal of external Na ions

The LRM preparation showed a slow development of tension in response to the removal of external Na ions (Fig. 4C). This response was about 20% of the maximum K-contractures, and was inhibited in Ca-free solution or in the presence of Mn ions (10 mM) indicating that the mechanical response may be due to Ca-influx caused by a possible Na–Ca exchange mechanism (e.g. Blaustein, 1976). An interesting feature concerning the mechanical response to Na-removal was its marked potentiating effect on ACh- and K-contractures. If ACh (10^{-3} M) was applied shortly (3–5 min) after the termination of the mechanical response to Na-removal, the resulting ACh-contracture tension was more than 50% larger than that for control ACh-contractures (Fig. 4D). Similar results were obtained with K-contractures, though the degree of potentiation was less pronounced.

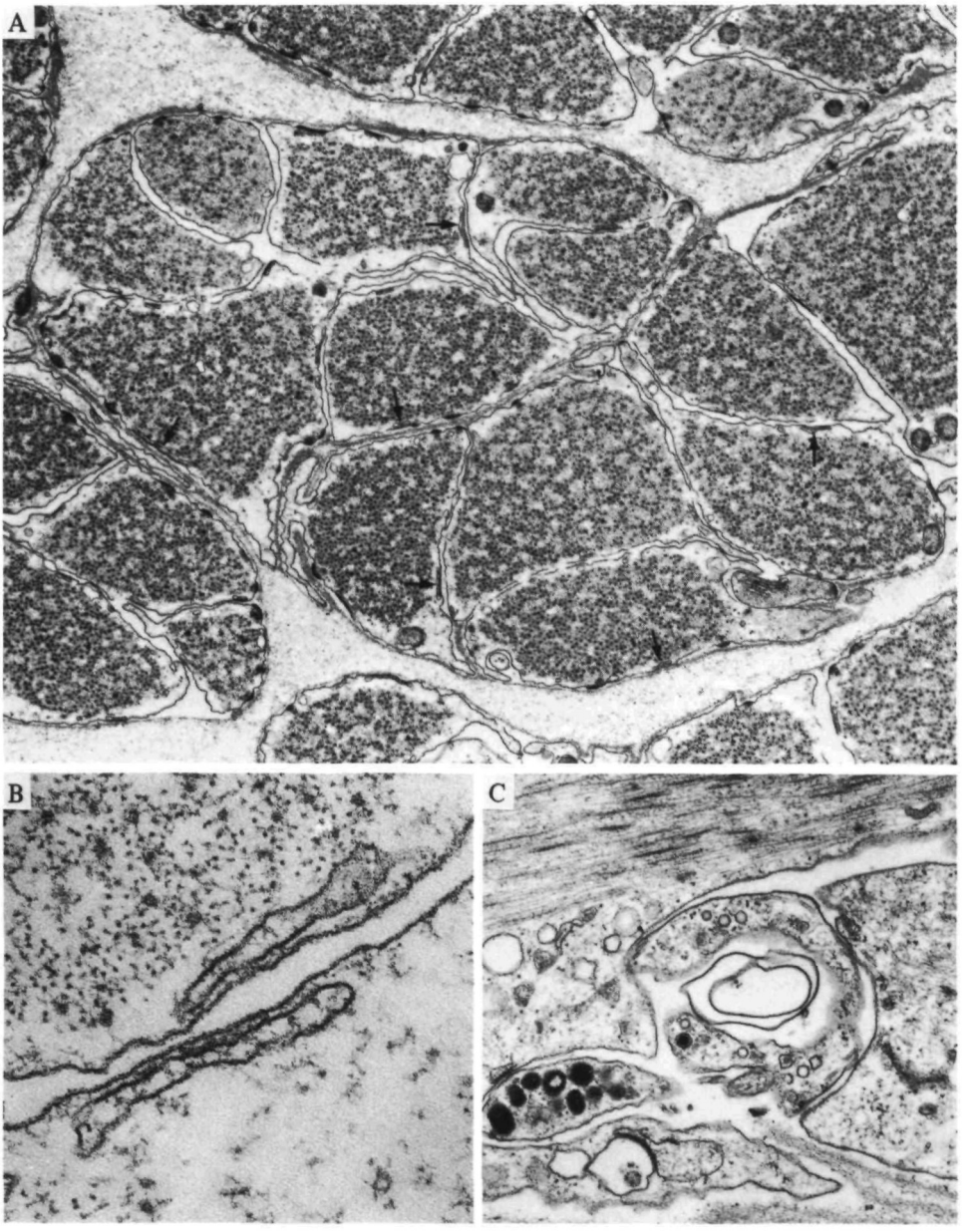

Fig. 5. Ultrastructure of the LRM. (A) Low-magnification electron micrograph of the LRM in transverse section showing individual muscle fibres with conspicuous projections. Note that the plasma membrane has no distinct invaginations, and small subsarcolemmal vesicles (arrows) are located underneath the plasma membrane (× 9300). (B) High-magnification view of a subsarcolemmal vesicle which is closely apposed to the plasma membrane with a distance of about 10 nm (× 77800). (C) Longitudinal section showing a neuromuscular junction. The nerve terminal is closely apposed to the fibre surface and contains large and small synaptic vesicles (× 19900).

Hypertonic solutions

When hypertonic solutions having an osmolarity about 2·0 times that of the standard solution were applied to the LRM preparation, it showed very gradual tension development irrespective of whether the hypertonic solutions were prepared by adding 613 mM NaCl or 1080 mM sucrose according to the formula used by Dydynska & Wilkie (1963) (Fig. 4E). The mechanical response to hypertonic solutions was reversible, and could be repeated many times. The magnitude of the response (10–20% of the maximum K-contractures) was not affected by the removal of external Ca ions even in the presence of EGTA (5 mM), but was reduced by 30–50% by procaine (5 mM).

Rapid cooling

Rapid cooling is known to produce contraction in various types of striated and smooth muscles (e.g. Sakai & Kurihara, 1974). In the LRM, however, rapid cooling of the standard solution from 20 to 5–2 °C within 5–10 s did not produce any detectable mechanical response.

Structure of the LRM fibres

Muscle fibre dimensions and interfibre relationships

The LRM is composed of many fibre bundles, each of them containing several muscle fibres running in the long axis of the muscle (Shida, 1971). The muscle fibres are uninucleate and unstriated with diameters of 2–5 μm and lengths of more than 200–300 μm. Fig. 5A shows the cross-section of the LRM fibres. The fibres contain thick (diameter, about 50 nm) and thin (diameter, about 8 nm) myofilaments, and the adjacent plasma membranes were frequently closely apposed with an interfibre space of 60–80 nm. The tight couplings between the adjacent fibres with a much smaller interfibre space were not observed, though such tight couplings have been reported to exist in the LRM of *Isostichopus badionotus* (Hill *et al.* 1978). The LRM fibres exhibit conspicuous cellular projections, which extend for a considerable distance as described by Hill *et al.* (1978) for the LRM of *I. badionotus*. The cellular projections extending from several fibres sometimes run in parallel with each other to form tight couplings between them.

Intracellular membranous structures

The LRM fibres show no distinct invagination of the plasma membrane, such as caveolae, and contain no well-developed intracellular membranous structures such as the SR. The only membranous structures frequently observed were small flattened vesicles located underneath the plasma membrane. As shown Fig. 5B, the vesicles are closely apposed to the plasma membrane at a distance of about 10 nm. These vesicles will be called the subsarcolemmal vesicles in this and the following paper (Suzuki & Sugi, 1982). Besides the subsarcolemmal vesicles, vesicles with much larger size were also seen much less frequently. Such apparent vesicles are, however, likely to form parts of the cellular projections, and are not true intracellular structures.

Neuromuscular junction

Motor nerve terminals were frequently observed along the length of the muscle fibres (Fig. 5C). They were closely apposed to the fibres with a gap of 20–50 nm, and contained two kinds of synaptic vesicles; small vesicles of 30–50 nm diameter and large cored vesicles of about 100 nm diameter. As with vertebrate smooth muscles (Burnstock, 1970), the postjunctional membrane of the LRM fibres did not show specializations found in vertebrate skeletal muscle, such as the formation of junctional folds and the thickening of the postjunctional membrane.

DISCUSSION

Activation of the contractile mechanism by ACh and high $[K]_0$

The present experiments have shown that ACh- and K-induced contractures in the LRM of *Stichopus japonicus* exhibit various features in common. The height of both ACh- and K-contractures was dependent on $[Ca]_0$, and both contractures were eliminated in Ca-free solution (Fig. 2), indicating that both types of contractures are associated with the inward movement of extracellular Ca ions. ACh- and K-contracture tension was, however, not markedly decreased by Mn ions, which block Ca-influx in various types of excitable membrane (e.g. Hagiwara & Nakajima, 1966) (Fig. 3A, B), and was unaffected by low pH, which inhibits Ca-influx in vertebrate smooth muscles (van Breeman *et al.* 1973). On the other hand, the height of ACh- and K-contractures was reduced to some extent in the presence of procaine (Fig. 3C, D), which is known to inhibit Ca-release from the SR by caffeine in vertebrate skeletal muscle (Feinstein, 1963; Weber & Herz, 1968).

These results suggest that, in the LRM, ACh and high $[K]_0$ produce not only the inward movement of extracellular Ca, but also the release of intracellularly stored Ca. On this basis, the long-lasting inhibitory effect of procaine on ACh-contractures may be explained as being due to its intracellular sites of action, and the accelerating effect of high $[K]_0$ on the recovery of ACh-contractures (Fig. 3E) may be due to a K-induced Ca-influx leading to an increase in the amount of intracellularly stored Ca that can be released by ACh. The observation that ACh-contractures are markedly enhanced after the mechanical response to Na-removal (Fig. 4D) may also be accounted for by an increased amount of intracellularly stored Ca resulting from the Ca-influx and the inhibition of its removal by the Na–Ca exchange mechanism. The abolition of ACh- and K-contractures in Ca-free solution may be taken to imply that the release of intracellularly stored Ca by ACh or high $[K]_0$ requires some loosely bound Ca at or near the fibre membrane.

The inhibitory effect of procaine was more marked in ACh- than in K-contractures (Fig. 3C, D), while that of Mn ions was more pronounced in K- than in ACh-contractures (Fig. 3A, B). These results, together with the marked potentiation of ACh-contractures after the mechanical response to the removal of external Na (Fig. 4D) and also the accelerating effect of high $[K]_0$ on the recovery of ACh-contractures after the treatment with procaine (Fig. 4E), may be taken to indicate that ACh causes the release of intracellularly stored Ca more effectively than high $[K]_0$.

Presence of intracellularly stored Ca in the LRM

The presence of intracellularly stored Ca available for the activation of the contractile mechanism in the LRM has also been demonstrated in the present study. A mechanical response of the LRM could be produced by caffeine (Fig. 4A). Caffeine is known to cause the release of Ca from, and inhibits the uptake by, the SR (Weber & Herz, 1968), and the mechanical response to caffeine has generally been taken as evidence for the presence of intracellularly stored Ca (e.g. Sandow, 1965).

The LRM could also be made to contract by the removal of external Ca and Mg ions (Fig. 4B). The mechanical response to the removal of external Ca and Mg has already been reported for frog slow skeletal muscle (Irwin & Hein, 1963) and for molluscan smooth muscle (Sugi & Yamaguchi, 1976), and has been explained as being due to the release of Ca from the intracellular structures in the absence of external divalent cations (Sugi & Yamaguchi, 1976).

In addition, the mechanical response to the LRM could also be produced by hypertonic solutions (Fig. 4E), even in the absence of external Ca ions. Hypertonic solutions normally cause flaccidity and decrease in tension in various types of muscle (Caputo, 1966; Sugi, Yamaguchi & Tanaka, 1977). Though the mode of action of hypertonic solutions on the LRM is at present obscure, a similar mechanical response to hypertonic solutions has been reported for frog fast skeletal muscle fibres (Länner-gren & North, 1973), and has been interpreted to result from the release of Ca from the SR.

These results indicate that the LRM fibres contain intracellularly stored Ca available for the activation of the contractile mechanism. The height of mechanical responses to caffeine, to the removal of external Ca and Mg and to hypertonic solutions was, however, not more than 30% of that of the maximum K-contractures with 10 mM-Ca, indicating that the total amount of activator Ca originating from the intracellular structures may not be enough to activate fully the contractile mechanism in the LRM.

Possible intracellular sources of activator Ca in the LRM

The LRM has poorly developed intracellular membranous structures compared with other vertebrate and invertebrate smooth muscles (e.g. Somlyo & Somlyo, 1976; Sugi & Suzuki, 1978). Since the mechanical activity in muscle is controlled by membrane potential changes in normal physiological conditions, the intracellular Ca-accumulating structures should be located close to the plasma membrane in order that they are electrically connected to the plasma membrane. The relatively rapid onset of mechanical response after the removal of external divalent cations (Fig. 4B) also indicates a close proximity of intracellular Ca-accumulating structures to the plasma membrane.

In this connexion, the surface vesicles in close apposition to the plasma membrane (Fig. 5A, B) are likely to be one of the sources of activator Ca (Huddart & Syson, 1975; Heumann, 1976). Another possible source of activator Ca is the inner surface of the plasma membrane, which has been shown to accumulate and release Ca during the contraction–relaxation cycle in some vertebrate and invertebrate smooth

muscles (Atsumi & Sugi, 1976; Sugi & Daimon, 1977; Suzuki & Sugi, 1978). Evidence that these intracellular structures in the LRM actually serve as sources of activator Ca will be presented in the following paper (Suzuki & Sugi, 1982). It may be argued that calcium storage sites do not need to be close to the plasma membrane if the Ca-influx resulting from membrane depolarization can cause the release of intracellularly stored Ca. Such a Ca-induced Ca-release mechanism observed in skinned fibres (Ford & Podolsky, 1970; Endo, Tanaka & Ogawa, 1970) is, however, regenerative in character, and seems to be inconsistent with the gradation of contraction controlled by membrane potential changes (Costantin, 1975).

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